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14. ABSTRACT
. 1) A total of 133 NF1 cases have been collected in the database. Among them, 101 cases are from HFHS site. There are currently 10 individuals with a previous breast cancer diagnosis. However, the analysis has not yet been performed. 2) Two blood samples have been collected from individuals who developed breast cancer. Germline NF1 gene analysis will begin once more samples are collected. 3) Five breast cancer tumor blocks have been collected. Immunohistochemistry validation of the antibodies has been completed; however the analysis of the tumor blocks has not commenced. 4) After successful NF1 knockdown, phospho-ERK1,2 was not increased to reflect the activated Ras in human mammary cell line MCF10A. Primary cells were expected to undergo senescence when exposed to hyperactive Ras. Therefore a senescence assay was attempted to demonstrate this effect. Human mammary epithelial cells (HMEC) derived from breast reduction surgery were then employed. Early observation showed that NF1 knockdown HMEC had the same rate of doubling time as the other Ras activation models, i.e. RB1 and p53-RB1 knockdown cells. Yet again, upregulated Ras could not be demonstrated by phospho-ERK1,2 assay. HMEC may need a longer period of doubling in order to be used to test these again.

15. SUBJECT TERMS- NF1; breast cancer; signaling pathway; germline mutation; somatic mutation; FFPE; IHC; next generation sequencing; ion semiconductor sequencing; NF1 knockdown cells; senescence.

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Table of Contents

INTRODUCTION

Several reports from England and the United States have described increased breast cancer occurrence in women affected with Neurofibromatosis type 1 (NF1). This study aims at identifying an accurate incidence of breast cancer in this group of women in a multi-center collaborative environment. There are 4 specific aims. Aim 1 is to confirm the increased breast cancer risk in women with NF1. All the participating centers, Henry Ford Health System (HFHS), University of Alabama at Birmingham (UAB), Children's National Medical Center in D.C. (CNMC), and Johns Hopkins University (JHU), will review the medical records of women affected with NF1. Clinical data will be analyzed to determine whether there are clinical features associated with the increased risk for breast cancer. At the same time, women with a history of breast cancer will be recruited to donate blood and their archived tumor specimen. Aim 2 is to analyze the germline *NF1* gene in the subjects with history of breast cancer. The mutations identified will be analyzed for genotype-breast cancer correlation. Aim 3 is to determine if NF1 associated breast cancers have unique signaling pathways and molecular tumorigenesis characteristics. Aim 4 is to study the phenotype of NF1 knockdown in primary mammary epithelial cells. This study will provide information that will help in determining when and how to screen for breast cancer in this group of women. It will also shed light on the molecular mechanisms of breast cancer in *NF1* deficient human subjects.

Aim 1 Task 1 and Task 2a:
IRB approval, construction of the database, and NF1 case review and clinical medical history data input

Sites	Planned for year 1	Tasks completed	Plan for year 2
HFHS (Henry Ford Health System) as coordination center	• IRB approval; • Web based database construction.	• IRB has approved the protocol. • Electronic database has been constructed and is fully functional.	Complete clinical data information integration and analysis by *March 2013.*
HFHS as clinical data collection site	Complete data entry by 9-30-2012.	• IRB has approved the protocol. • 101 cases have been entered into database.	Continue to input additional data when new cases emerge.
UAB (University of Alabama at Birmingham)	• IRB approval; • Approximately 150 cases to be entered by 9-30-2012	• IRB has approved the protocol. • 17 cases have been entered into database thus far.	Complete the task by *February, 2013*.
***CNMC** (Children's National Medical Center)	• IRB approval; • Approximately 200 cases to be entered by 9-30-2012.	• IRB has approved the protocol. • 15 cases have been entered into database thus far.	Complete the task by *February, 2013*.
JHU (Johns Hopkins University)	• IRB approval; • Approximately 150 cases to be entered by 9-30-2012.	• JHU underwent 7 protocol revisions in response to IRB requests. The last revision was approved on 10-12-2012. • JHU has not yet entered cases into the database.	Complete the task by *February, 2013*

*The delay at the CNMC site is mainly due to the fact that this site only sees children in the clinic. CNMC has to recruit NF1 affected adult female family members by obtaining consent to gather medical history. The enthusiasm to participate was over-estimated.

Aim 1 Task 2c, 2d

Identify breast cancer cases in NF1 women, recruit them to participate in the study, and collect blood and tumor specimens

Sites	• Planned for year 1	• Tasks completed	• Plan for year 2
HFHS	• Start to contact participants for consent and specimen collection by July 2012.	• 6 tumor blocks collected. • 2 blood specimens collected.	• Continue to identify subjects; recruit and collect specimens.
UAB	• Start to contact participants for consent and specimen collection by July 2012	• None has been collected.	• Plan to recruit tumor specimens and blood specimens for 10 cases by *June, 2013*, as planned in the SOW.
CNMC	• Start to contact participants for consent and specimen collection by July 2012	• None has been collected	• Plan to recruit tumor specimens and blood specimens for 5 cases by *June, 2013*, as planned in the SOW. • Effort has been made to advertise the recruitment to local NF organization.
JHU	• Start to contact participants for consent and specimen collection by July 2012	• None has been collected.	• Plan to recruit tumor specimens and blood specimens for 10 cases by *June, 2013*, as planned in the SOW. • 4-6 tumor blocks are expected to be collected from the clinic patient pool. Additional cases may be collected through communication with other departments in JHU.

Aim 2

Germline NF1 gene analysis on individuals affected with breast cancer

Sites	Original plan for year 2	Tasks completed	New plan for year 2
HFHS	*NF1* gene analysis on 10 blood specimens to be completed by Jan 1, 2013	• 2 blood specimens have been collected. • No *NF1* gene analysis has been completed.	• *NF1* gene analysis on 10 blood specimens to be completed *by Jan 1, 2013* • *NF1* gene mutation data integration and analysis to be completed by *June, 2013*, as planned in the SOW.
UAB	*NF1* gene analysis on 20 blood specimens to be completed by Jan 1, 2013	Not yet completed.	*NF1* gene analysis on 10 blood specimens to be completed by *Jan 1, 2013*
CNMC	*NF1* gene analysis on 10 blood specimens to be completed by Jan 1, 2013	Not yet completed.	*NF1* gene analysis on 5 blood specimens to be completed by *Jan 1, 2013*
JHU	*NF1* gene analysis on 20 blood specimens to be completed by Jan 1, 2013	Not yet completed.	*NF1* gene analysis on 10 blood specimens to be completed by *Jan 1, 2013*

Aim 3 Task 4a

DNA molecular analysis for loss of heterozygosity (LOH) and methylation on formalin fixed paraffin embedded (FFPE) tumor tissue

Site	Planned for year 1	Tasks completed	Plan for year 2
HFH Pathology	Assay validation for LOH, methylation with MLPA technique by September, 2012.	This task has not been started yet because of the short storage time for reagents.	Assay validation will be carried out once all the FFPE tumor specimens are collected.
			Complete MLPA assay and data analysis by *September, 2013*, as planned in the SOW.

***Recommend changes**			Sequencing and copy number analysis of 22 genes, including *NF1* gene, utilizing ion semiconductor sequencing technology on FFPE tumor specimens

*Details about the recommended changes are described on page 22.

Aim 3 Task 4a

Immunohistochemistry (IHC) analysis for signaling pathway on formalin fixed paraffin embedded (FFPE) tumor tissue

	Planned for year 1	**Tasks completed**	**Plan for year 2**
HFH Pathology	Complete IHC assay validation with antibodies against signaling proteins by September, 2012.	Validation has been completed.	
			Complete assays on all FFPE specimens and complete data analysis by *September, 2013*, as was planned in the SOW.
***Recommend changes**			Add Ki 67 IHC assay in addition to assays for MEK, ERK, pERK, Akt, mTOR, HER2, p53, and PTEN

*Details about the recommended changes are described on page 22.

Details on Aim 3 Task 4a:

To determine if NF1-associated breast cancers have unique signaling pathway and molecular characteristics.

Progress of validation of immunohistochemistry assays for p53, mTOR, PTEN, Phospo-MEK 1/2 (Ser221), Phospo-p44/42 MAPK (ERK1/2), P44/42 MAPK (Erk 1/2), AKT (pan), HER2/ERB2

Tissue selection for validation:

To explore the oncogenic pathway of Ras signaling and PI3K pathway activation by immunohistochemistry (IHC) analysis, validations were performed on 22 formalin fixed paraffin embedded (FFPE) tissues. These included 15 known sporadic de-identified archival breast cancer tumor specimens and 7 normal breast parenchymal tissues. The tumor tissues were selected to include known estrogen receptor and progesterone receptor positive tumors, Her2 positive tumors and triple negative tumors. Tissue microarray was constructed using manual Beacher instrument and all the sections were cut at 4u thickness. H and E stained sections were examined to confirm presence of tumor and normal tissues.

Antibody selection:

All the antibodies were obtained from the Cell signaling as written in the application. IHC staining for pMEK (the phosphorylated and activated form of MEK), ERK (the non-phosphorylated ERK) and pERK (the phosphorylated and activated ERK) proteins was performed. To examine PI3K pathway activation, IHC for pAKT (phosphorylated and activated AKT) and mTOR protein was done. Antibodies against p53, PTEN and HER2 proteins were also validated by IHC.

Validation process:

The tissue microarray sections were mounted on charged slides, deparaffinized, and rehydrated using standard immunohistochemistry protocol. In all, eight antibodies listed above were run with three different dilutions of the primary detection antibodies, including one manufacturer recommended concentration (*). Two different antigen retrieval methods were employed to fine tune the best possible staining pattern (nuclear or cytoplasmic) viz.
1. Envision FLEX target retrieval solution Low pH, citrate pH 6.1 and
2. Envision FLEX target retrieval solution High pH, Tris/EDTA pH 9.0.

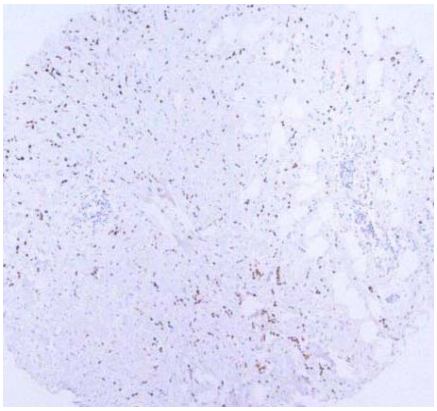
Signal detection was performed using anti-Rabbit antibody (Rabitt FLEX+) and DAB chromogen.

Total of 352 tissue core sections were evaluated [22 samples x 8 antibodies x 2 antigen retrieval methods = 352).
Based on the best staining pattern, corresponding dilution and antigen retrieval are selected and ready for second phase of the study to stain all of the breast cancer tumors from the recruited NF1 patients. Detailed validation charts are listed below with pertinent examples of tumor microphotographs.

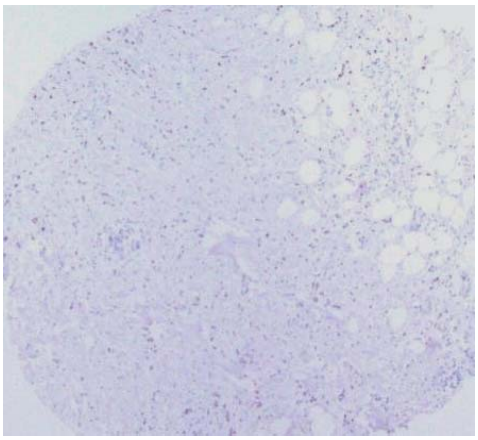
1. TP53:

P53 (75F) Rabbit mAB (Catalog #2527): Nuclear staining

Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:40	TRL	20 min	Rabbit Flex+	DAB+	
1:40*	TRH	20 min	Rabbit Flex+	DAB+	X
1:80	TRL	20 min	Rabbit Flex+	DAB+	
1:80	TRH	20 min	Rabbit Flex+	DAB+	
1:160	TRL	20 min	Rabbit Flex+	DAB+	
1:160	TRH	20 min	Rabbit Flex+	DAB+	



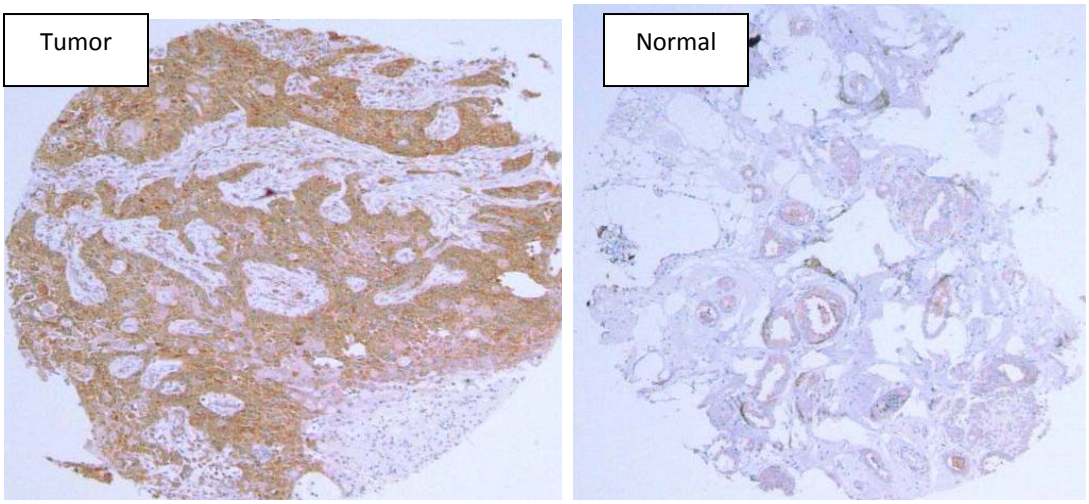
1: 40, TRH, crisp nuclear staining



1:160, TRH, weak staining of same tumor

2. mTOR (7C10) Rabbit mAB (Catalog #2983): cytoplasmic staining

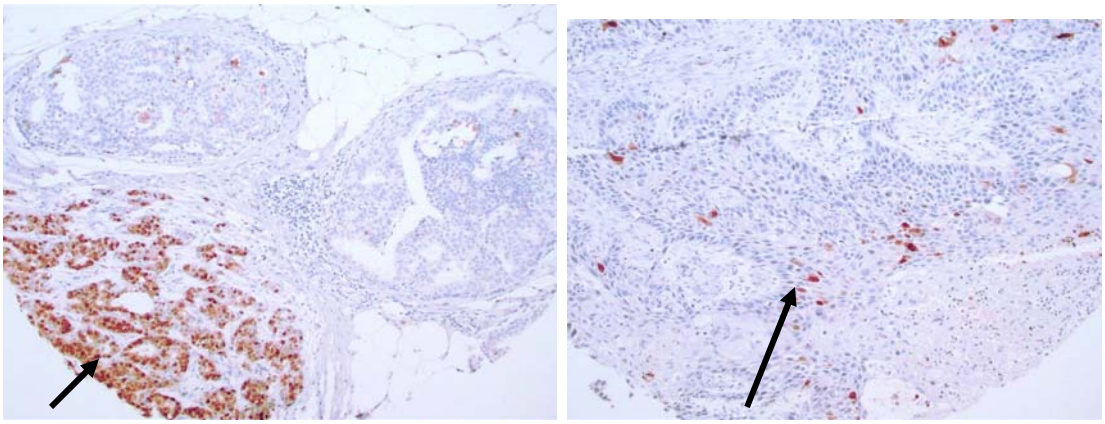
Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:50*	TRL	20 min	Rabbit Flex+	DAB+	
1:50	TRH	20 min	Rabbit Flex+	DAB+	X
1:100	TRL	20 min	Rabbit Flex+	DAB+	
1:100	TRH	20 min	Rabbit Flex+	DAB+	
1:160	TRL	20 min	Rabbit Flex+	DAB+	
1:160	TRH	20 min	Rabbit Flex+	DAB+	



Cytoplasmic staining of tumor (strong) and normal (weak)

3. Phospo-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP™ Rabbit mAB (Catalog #4370): Nuclear

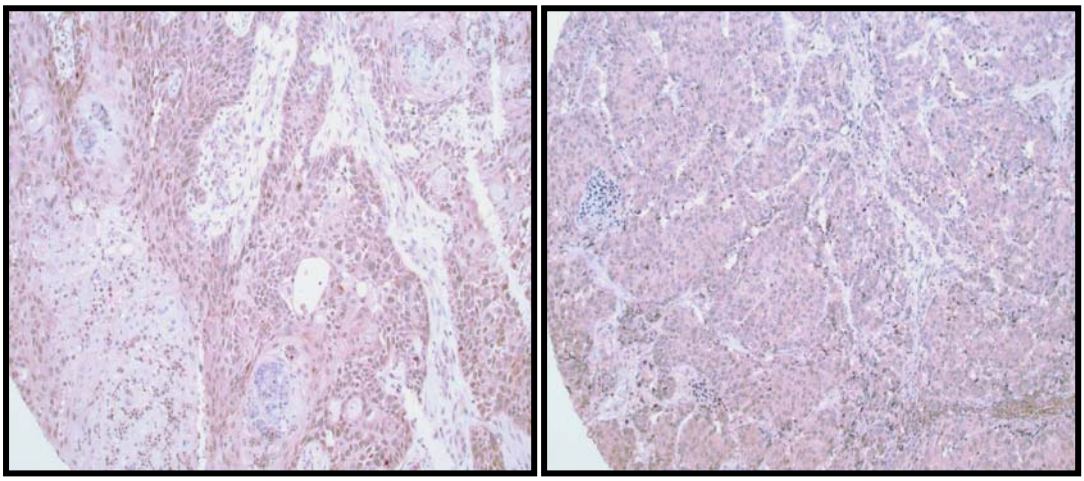
Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:50	TRL	20 min	Rabbit Flex+	DAB+	
1:50	TRH	20 min	Rabbit Flex+	DAB+	
1:100	TRL	20 min	Rabbit Flex+	DAB+	
1:100	TRH	20 min	Rabbit Flex+	DAB+	
1:300*	TRL	20 min	Rabbit Flex+	DAB+	
1:300	TRH	20 min	Rabbit Flex+	DAB+	X



Strong nuclear staining in the invasive carcinoma vs focal nuclear staining in another case

4. Phospo-MEK 1/2 (Ser221) (166F8) Rabbit mAB (Catalog #2338)

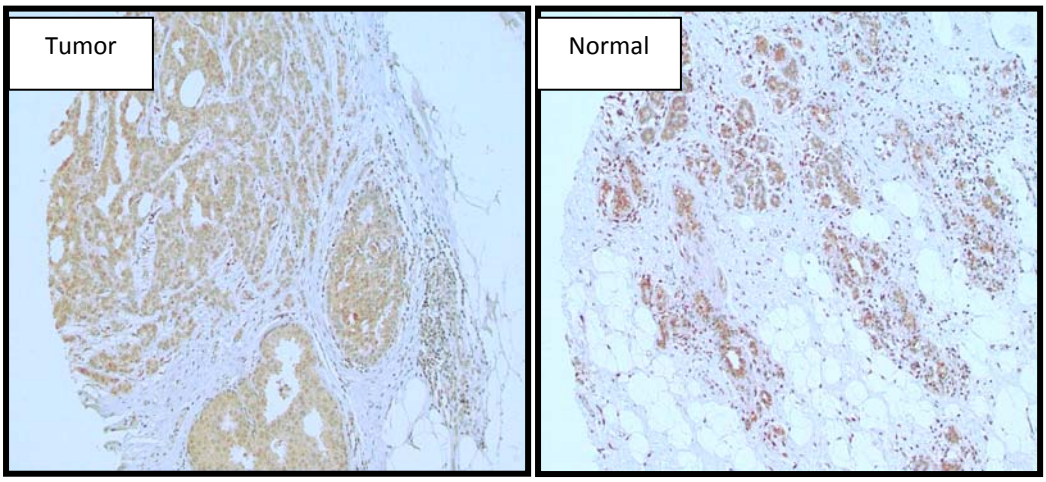
Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:50*	TRL	20 min	Rabbit Flex+	DAB+	
1:50	TRH	20 min	Rabbit Flex+	DAB+	X
1:100	TRL	20 min	Rabbit Flex+	DAB+	
1:100	TRH	20 min	Rabbit Flex+	DAB+	
1:160	TRL	20 min	Rabbit Flex+	DAB+	
1:160	TRH	20 min	Rabbit Flex+	DAB+	



Two tumors with cytoplasmic and focal nuclear staining

5. PTEN (D4.3) Rabbit mAB (Catalog #9188)

Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:125*	TRL	20 min	Rabbit Flex+	DAB+	
1:125	TRH	20 min	Rabbit Flex+	DAB+	
1:250	TRL	20 min	Rabbit Flex+	DAB+	
1:250	TRH	20 min	Rabbit Flex+	DAB+	X
1:500	TRL	20 min	Rabbit Flex+	DAB+	
1:500	TRH	20 min	Rabbit Flex+	DAB+	

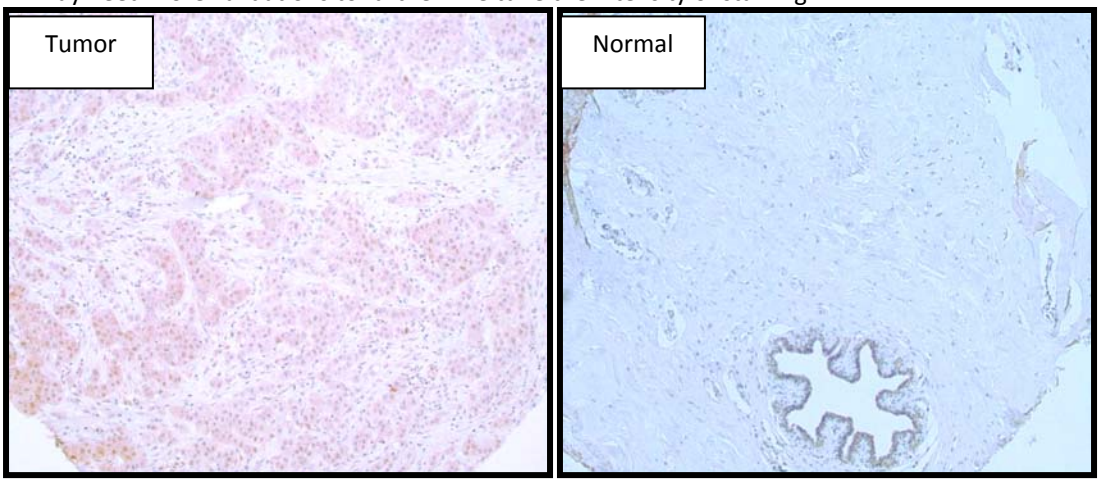


Cytoplasmic staining of tumor and normal mammary lobules

6. AKT (pan) (11E7) Rabbit mAB (Catalog #4685)

Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:100*	TRL	20 min	Rabbit Flex+	DAB+	
1:100	TRH	20 min	Rabbit Flex+	DAB+	X **
1:300	TRL	20 min	Rabbit Flex+	DAB+	
1:300	TRH	20 min	Rabbit Flex+	DAB+	
1:500	TRL	20 min	Rabbit Flex+	DAB+	
1:500	TRH	20 min	Rabbit Flex+	DAB+	

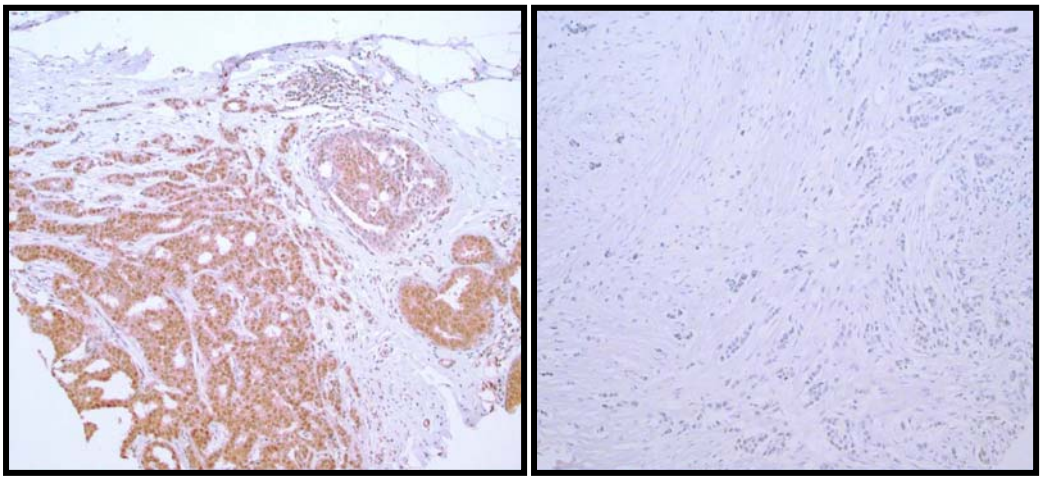
** may need more validations to further fine tune the intensity of staining



Akt1 was localized predominantly in the cytoplasm (the left side- in tumor cells) and weakly positive in normal mammary ducts.

7. P44/42 MAPK (Erk 1/2) (137F5) Rabbit mAB (Catalog #4695)

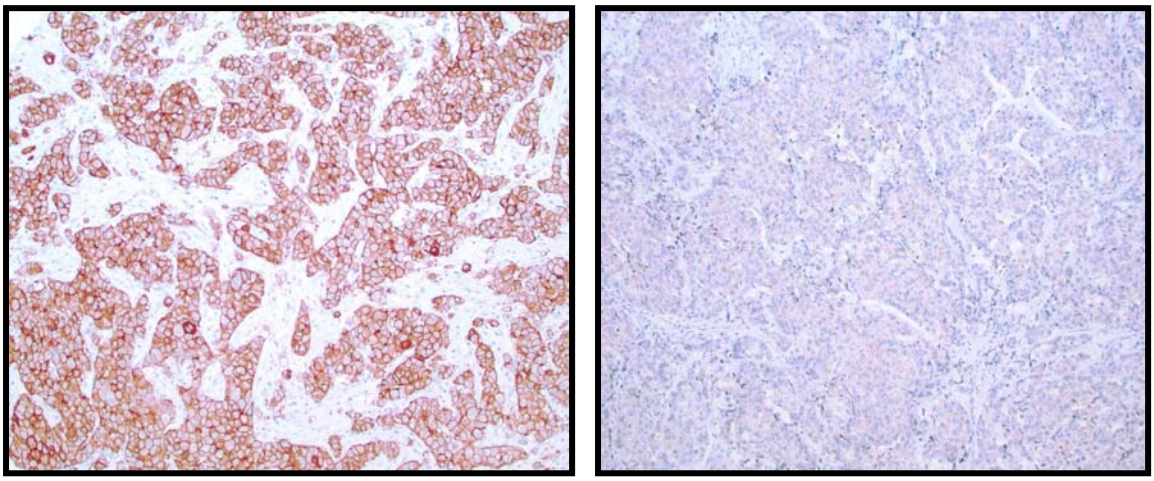
Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:250*	TRL	20 min	Rabbit Flex+	DAB+	
1:250	TRH	20 min	Rabbit Flex+	DAB+	
1:500	TRL	20 min	Rabbit Flex+	DAB+	
1:500	TRH	20 min	Rabbit Flex+	DAB+	
1:1000	TRL	20 min	Rabbit Flex+	DAB+	
1:1000	TRH	20 min	Rabbit Flex+	DAB+	X



Example of strong positive nuclear and cytoplasmic localization in invasive and in-situ carcinoma (left). Another example with complete negative staining on the right.

8. HER2/ERB2(29D8) Rabbit mAB (Catalog #2165)

Antibody Dilution	Antigen retrieval	Primary incubation time	Detection system	Detection chromogen-amplification	Selected method
1:400	TRL	20 min	Rabbit Flex+	DAB+	
1:400*	TRH	20 min	Rabbit Flex+	DAB+	X
1:800	TRL	20 min	Rabbit Flex+	DAB+	
1:800	TRH	20 min	Rabbit Flex+	DAB+	
1:1200	TRL	20 min	Rabbit Flex+	DAB+	
1:1200	TRH	20 min	Rabbit Flex+	DAB+	



Strong membranous 3+ positive staining on left and negative staining on the right.

Conclusion:
All the eight antibodies have been validated and are ready to use on the breast cancer FFPE tumor blocks from the NF-1 patients.

Details on Aim 4

Phenotypic Analysis of NF1 Knockdown in Normal Mammary Epithelial Cells

Summary of Tainsky Lab work in year 1

MCF10A, an established cell line representing normal mammary epithelial cells, was infected with a lentiviral knockdown system for neurofibromin, shRNAmir-NF1 (Openbiosystems, ThermoFisher Scientific). Neurofibromin expression was determined to be suppressed in the knockdown population which resulted in 5.5-fold decreased expression of the protein as compared to controls. Microscopic examination of green fluorescent protein expression indicated 90-95% infection. However phospho-ERK1,2, a downstream Ras signaling factor, remained unchanged and differences in population doubling (PD) capacity were not observed. It was concluded that the immortal cell line had acquired transformation characteristics making it insensitive to the biochemical effects of loss of a functional neurofibromin such as hyper-activated Ras and a variety of tumorigenic characteristics. In order to continue this important line of research it was decided to use normal primary mammary epithelial cells to observe the early events after a knocking down neurofibromin expression using the lentiviral technology.

Normal Human Mammary Epithelial Cells (HMEC), (Invitrogen, Inc) originally obtained from breast reduction mammoplasty, were used for further experiments. It has been widely reported that some human primary cells, among them HMECs, undergo senescence when exposed to a mutated/hyperactive oncogene such as Ras. Ras downstream activity is quickly upregulated followed by a negative feedback response that induces senescence. This oncogene induced senescence (OIS) is thought to be a protective mechanism against tumorigenicity. We performed experiments to determine whether this mechanism could be induced in HMECs by an *NF1* deficiency. RB1 and p53 pathway involvement in the senescence response was also examined as these pathways have been implicated in other cell types. The HMECs were infected with vectors for the knockdown NF1 RNA or non-silencing RNA as well as additional knockdowns; p53, RB1, and p53+RB1. The number of PDs were determined and senescence-associated beta-galactosidase assays were performed at each passage during a 45 day growth period. See Table 1.

Table 1. Total population doubling over 45 day experimental period

sample	total population doubling
NF1+p53	-0.07
NF1+RB1	2.57
NF1+RB1+p53	2.68
NF1+NON	2.74
NON+NON	4.16
Where NON+NON are the control cells	

Growth of knockdown NF1 cells was slowed by about 35% as compared to non-silencing RNA cells (NON+NON) while the NF1 knockdown populations showed about 3.6-fold increase in senescent cells. The number of PDs or fraction of cells in senescence was not different in the NF1+NON, NF1+RB1 or NF1+p53+RB1 knockdown populations. These results suggest that an **NF1 deficiency does initiate OIS** independent of RB1 or a combination of p53 and RB1 pathways.

Protein expression during the 45 day growth period was also evaluated. In parallel, samples were collected for western blotting indicative of proteins related to senescence, i.e., p21, p16, RB1, p53 as well as Ras pathway

signaling, phosphorylated ERK and total ERK. Under conditions of the experiment the expressions of NF1 p53, and RB1 knockdown and control cell populations were not different which within the sensitivity of the assay knockdowns did not occur. In addition expression of phosphorylated ERK1,2 were also not different in the control and NF1 knock out populations indicating that the Ras pathway had not been upregulated. Although some effect was seen in the senescence and PD assays, we believe that the conditions for lentiviral infections needs to be optimized. It is possible that optimization would take a high number of PDs. However, crisis occurs within 16 PDs in these cells inducing a senescence from which the cells cannot escape. This limited greatly our ability to experiment with the cells. In addition it may be that targeted inhibitors rather than knockdowns would shorten the length of the experiments but drugs also need to be optimized for suitable concentration levels. The senescence and growth patterns of the cells were very encouraging and we believe that additional experimentation would yield important information regarding OIS. The current grant budget did not allow for the supplies and salaries ($20-$25,000) to continue in this project.

Figure 1:

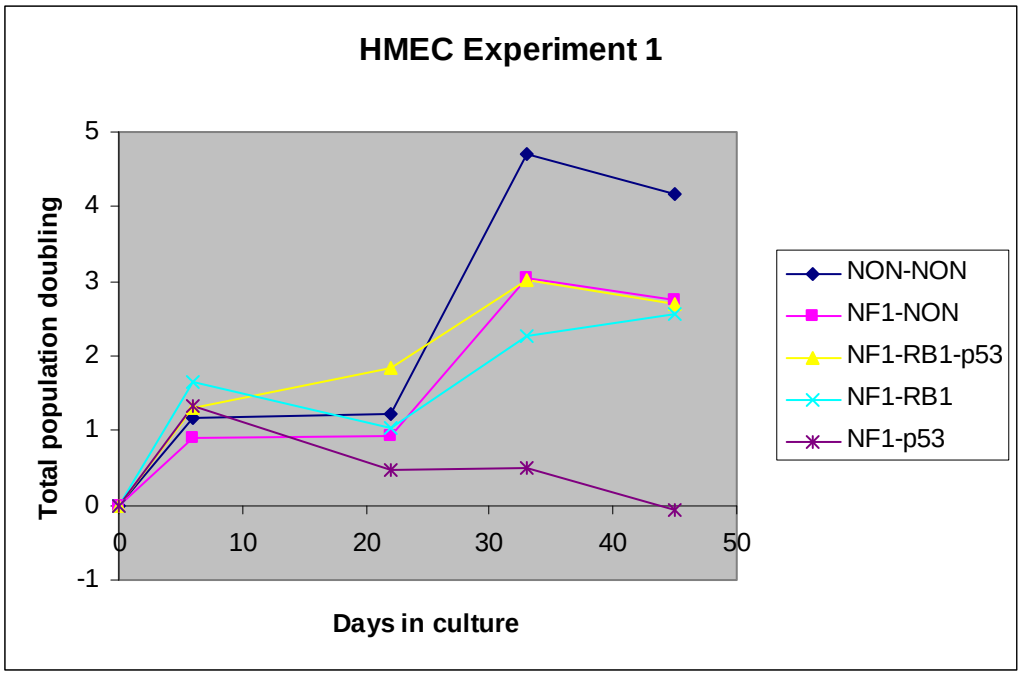
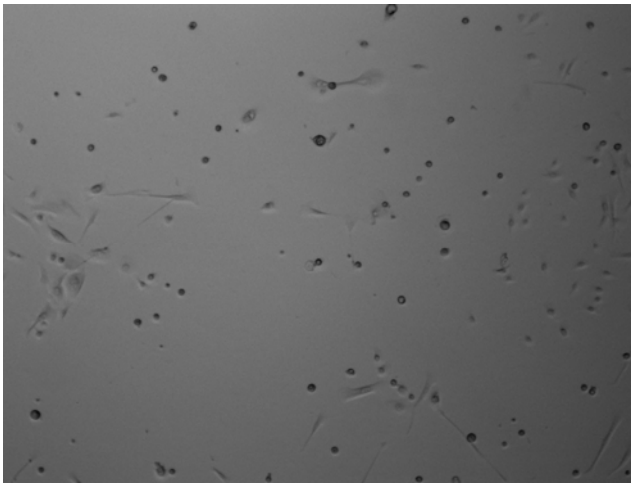
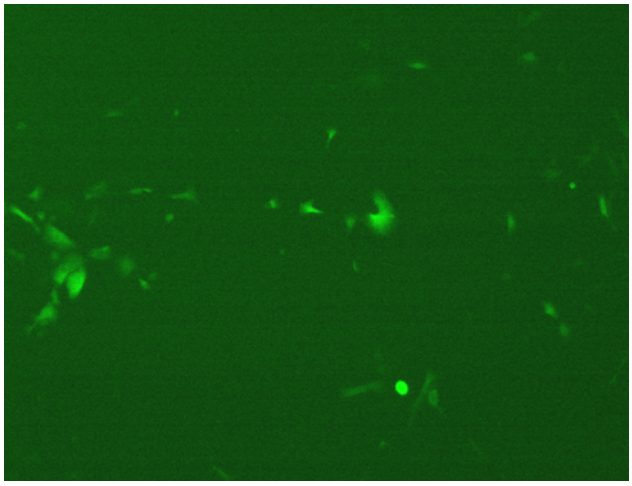


Figure 2: Ten days after infecting HMECs with shRNAmir-NF1 knockdown and 6 days after adding a selection agent for plasmid expression. Of the cells that had been seeded and spread out, 95 to 100% demonstrated green fluorescent protein expression, see figures below.

Similar results were found for infection of non-silencing RNA, i.e., control cells





file: p/nf1/xai grant/gfp images/7-16-12

KEY RESEARCH ACCOMPLISHMENTS

None

REPORTABLE OUTCOMES

None

CONCLUSION AND RECOMMEND CHANGES

No conclusion can be drawn at this time as data collection and recruitment is still ongoing. Germline *NF1* gene analysis and FFPE tumor specimen testing will be performed once all samples have been collected. However, recommended changes are described here. Due to the recent availability of next generation sequencing technology, reliable full gene sequencing on FFPE tumor specimen has become reality. We plan to sequence the *NF1* gene and test copy number variation by ion semiconductor sequencing technology – Ion torrent. Ion torrent is better suited for low sample quantity. The cost is also relatively low. If bi-allelic *NF1* gene mutation or deletion is detected in most of the tumors, it will confirm the double hit oncogenesis hypothesis for the breast cancer in NF1 women. If only a single allele mutation or deletion is found in the tumor and is concordant with the germline *NF1* mutation, the double hit mechanism cannot be established. We then need to look for the evidence of other genetic changes.

Based on the recent published breast cancer comprehensive genomic information[1], we plan to add additional gene analysis targeted at the genes bearing somatic mutations or deletions in the sporadic breast cancers. In addition to *NF1, BRCA1, BRCA2, TP53, PTEN*, and *ATM* genes, we plan to analyze *CDH1, RB1, MLL3, MAP3K1, CDKN1B, PIK3CA, AKT1, GATA3 TBX3, RUNX1, CBFB, AFF2, PIK3R1, PTPN22, PTPRD, 3F3B1*, and *CCND3* genes on the FFPE tissue. We will use ion semiconductor sequencing and MLPA technology to achieve this goal.

Since Ki 67 is an integral part of breast cancer classification, we plan to add IHC assay for this marker, in addition to MEK, ERK, pERK, Akt, mTOR, HER2, p53, and PTEN.

REFERENCES

1. The Cancer Genome Atlas Network, “Comprehensive molecular portraits of human breast tumours”, 2012, Nature 490(7418):61-70

APPENDICES

None